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Detection of virulence factors of Mycoplasma species isolated from chicken by multiplex PCR

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ABSTRACT

One hundred and sixty clinically diseased broiler local chickens collected from 22 different farms belonging to Qalyubia, Dakahlia and Gharbia governorates were subjected to bacteriological examination and molecular characterization. Clinical signs were chronic respiratory disease with respiratory manifestations, lameness, loss or reduction in egg production. Bacteriological examination showed that 18.18% of the isolates were *Mycoplasma* positive and showed growth in pleuropneumonia-like organism(PPLO) agar plates, with fried egg appearance when examined by stereoscopic microscope. *Mycoplasma* colonies were tested for antimicrobial sensitivity tests against 12 antimicrobial antibiotics showed higher sensitivity to nitrofurantoin, gentamicin, norfloxacin, ciprofloxacin and neomycin. Intermediate sensitivity were recorded to ceftriaxone and cefotaxime, and antibiotic resistance was recorded to tetracyclines, lincomycin, chloramphenicol and sulphamethoxasine. Identification of the bacterial strains of the isolates was conducted by multiplex PCR using two primer pairs for *Mycoplasma synoviae* (*v*lhA) and *Mycoplasma gallisepticum* (*mgc*2). The amplicons expected sizes were 396 bp, and 300 bp for *v*lhA and *mgc*2, respectively. Only13 out of 22 farms were positive, representing 59.09 %.Moreover, the incidence rate of *M. synoviae* (*vlh*A) and *M. gallisepticum* (*m*gc2) was22.72% and 13.63%, respectively, and 22.72% of the inspected farms showed positive results for both *Mycoplasma* strains. In conclusion, High prevalence of mixed M. *synoviae* and M. *gallisepticum* infections in poultry cause respiratory manifestations. Multiplex PCR is sensitive and specific for simultaneous detection of *M. synoviae* and *M. gallisepticum* in a single reaction.

1. INTRODUCTION

Mycoplasma is a member of *Mollicutes class*. *Mycoplasma* is recognized as the most important pathogen in poultry associated with high mortality rates, increase in carcass condemnations and drop in egg production(Levisohn and Kleven, 2000).Moreover, transient suppression of humeral and cellular immune responses, immune tolerance and auto immune diseases, as well as the massive lymphoid cell infiltration in the respiratory tract and joint tissues of infected fowls were occurred during *mycoplasma* infection(Yamamoto et al., 1990; Razin et al., 1998).*Mycoplasmas* infection is induced after the host is exposed to stress factors like vaccination, cold weather, overcrowding, feed/water restriction, temperature extremes, poor ventilation and other stress. *Mycoplasma* infection is usually associated with respiratory manifestations, high mortalities, reduced weight gain and condemnation of birds at the slaughter.

Mycoplasma gallisepticum strain was first isolated by Yoder (1980), while *M. synoviae* was first isolated from synovial sheath of commercial chickens by Morrow et al. (1990).

The most important *Mycoplasma* pathogens of the poultry are *Mycoplasmagallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis* (only for turkeys) and *Mycoplasma iowae*). All of them causes significant economic losses *(Kleven, 1997; Ley* 1997*; Yoder, 1991).*

Mycoplasmas are thought to colonize in mucosal surfaces more efficiently and become more virulent by alternating the composition of their surface proteins. *Mycoplasma gallisepticum* cytadhesin membrane surface proteins that undergo changes are represented by *p*mgaS (hemagglutinins), *m*gc1, *m*gc2 and *p*vpA (*Bencina et al. 2002). Mycoplasma Synoviae* has two major surface antigens, that are encoded by a single gene, *v*lhA(variably expressed lipoprotein(MSPB) and the haemagglutinin (MSPA)) (Kiseok et al., 2010). Recent comparison of the *M. gallisepticum* genome with the *M. synoviae* genome revealed that a number of their genes have been transferred horizontally (Papazisi et al., 2003).

Although antimicrobials are considered very important method for treatment of clinical disease and maintaining birds' health and productivity, they have been implicated as risk factors in the dissemination and development of drug resistance (Whithear et al. 1983). *M. gallisepticum* may

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develop resistance and even demonstrate cross-resistance to commonly used antibiotics(Zanella et al., 1998). Moreover, *Mycoplasma* revealed sensitivity to tetracyclines and quinolones, but they are resistant to penicillin and other antibiotic inhibitors of cell wall synthesis (Bébéar et al. 1999).

The aim of the present study was to evaluate a multiplex PCR assay for rapid detection of *Mycoplasma* pathogens in clinical specimens of chicken suffered from respiratory manifestations and loss of egg production thus would allow earlier and appropriate treatment as well as control of the diseases.

2. MATERIAL AND METHODS

2.1. Chicken samples:

A total of 160 chicken samples were collected aseptically from heart, liver, trachea and synovial fluids from 22 different farms suspected to be infected with *Mycoplasma* and belonging to Qalyubia, Dakahlia and Gharbia governorates.

2.2. Bacteriological examination:

Pleuropneumonia-like organism(PPLO) plus DNA media (PPLO broth or PPLO agar, Horse serum, Yeast extract 5% solution, DNA 0.2% w/v solution, Penicillin G-Sodium and Thallium acetate 2% w/v solution) wasused for bacteriological examination according to Sabry(1968).

2.3 Microscopical examination:

By using Geimsa staining technique as described by Sabry (1968) for morphological study.

2.4. Biochemical identification of the bacterial isolates:

It was performed according to Sabry (1968) including Glucose fermentation medium (Phenol red dextrose broth base, Horse serum, Thallium acetate 2% w/v solution and Penicillin G-Sodium) and Arginine deamination medium (Phenol red broth base, L-arginine solution (10% w/v solution), Horse serum, Thallium acetate 2% w/v solution and Penicillin G-Sodium)

2.5. Antimicrobial sensitivity test:

The disk diffusion method was applied according to Bauer et al. (1966). All *mycoplasma* isolates were tested for their antimicrobial susceptibility by 12 different antimicrobial agents (Difco™) including;neomycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), tetracycline (30 µg), ciprofloxacin (30 µg), gentamicin (10 µg), oxytetracycline (30µg), ceftriaxone (30 µg), lincomycin(µg), norfloxacin (10 µg), cefotaxime (30 µg), sulphamethoxasine (100 µg), chloramphenicol (30 µg). The interpretation of inhibition zones of tested culture was done according to NCCLS, (2002), when the zone of inhibition had a diameter ≥ 20mm, the isolate was considered sensitive to the used antibiotic.

2.6. Detection of virulence genes PCR method:

DNA extraction was performed using QIA amp DNA mini extraction kit according to the manufacturer's instruction. DNA was amplified by using the PCR method *(*Lysnyansky et al., 2005).

PCR Master Mix used for cPCR is Emerald Amp GT PCR mastermix (Takara) Code No. RR310A Contains Emerald Amp GT PCR mastermix(2x premix)and PCR grade water.Temperature and time conditions of the two primers during PCR are shown in table (1) according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

Table 1 Cycling conditions of the different primersduring cPCR.

Final extension	No. of cycles	Extension	Annealing	Secondary denaturation	Primary denaturation
72˚C 10 min.	35	72˚C 1 min.	55˚C 30 sec.	94˚C 30 sec.	94˚C 5 min.

For detection of the target genes (*vlha and mgc2*). PCR products were separated by gel electrophoresis and visualized using U.V trans-illuminator

3. RESULTS

3.1. Incidence of Mycoplasma in the examined chicken farms by using conventional culture method:

Out of 160 samples taken from diseased broiler chickens from 22 different broiler farms from Qalyubia, Dakahlia and Gharbia Governorates, 4 farms showed *mycoplasma* positive, with an incidence rate of 18.18%. Isolates appeared as fried egg when examined by stereoscopic microscope, sensitive to digitonin, positive to glucose fermentation test and negative for arginine deamination test.

3.2. Antimicrobial sensitivity testing of Mycoplasma isolated from broiler chickens:

Results of *in-vitro* antimicrobial sensitivity testing for *Mycoplasm a*isolates were demonstrated in table (3). *Mycoplasma* isolates were highly resistant to tetracyclines. Moderate sensitivity was observed against ceftriaxone and ciprofloxacin, and highly sensitive to gentamicin and nitrofurantoin.

Table 3 Antibiogram patterns for *Mycoplasma* recovered from cases of diseased broiler chickens and local breeds' chickens

Antibacterial agents	Disc content(µg)	(*n=4*)			
		Susceptible		Resistant	
		No.	%	No.	%
Neomycin	3	3	75	1	25
Ceftriaxone	30	2	50	2	50
Nalidixic acid	30	3	75	1	25
Ciprofloxacin	30	3	75	1	50
Tetracycline	30	0	0	4	100
Gentamicin	10	3	75	1	25
Nitrofurantoin	30	3	75	1	25
Lincomycin	10	2	50	2	50
Chloramphenicol	30	2	50	2	50
Sulphamethoxasine	100	2	50	2	50
Norfloxacin	10	3	75	1	25
Cefotaxime	30	2	50	2	50

No.: Number of positive cases.%: was calculated according to the total number of *mycoplasma* isolat*es* (n=4)

3.3. Incidence of Mycoplasma species in different poultry farms by using multiplex PCR were demonstrated in table (4)

3.4 Comparison between percentage of detection of mycoplasma from clinical samples using conventional culture methods and PCR in different governorates were demonstrated in table (5)

Table 4 Incidence of Mycoplasma species in different poultry farms by using multiplex PCR

Bacterial infections	No. of infected farms (%)	Type of production	
		Local breed	broiler
M. gallisepticum	3 (13.63%)	3	0
M. synoviae	5 (22.72%)	4	1
M.gallisepticum and *M.synoviae*	5 (22.72%)	4	1
No growth	9(40.9%)	5	4
Total	22 (100%)	16	6

Table 5 Comparison between percentage of detection of mycoplasma from clinical samples using conventional culture methods and PCR in different governorates

Incidence of Mycoplasma farms		Conventional culture methods	PCR
Governorate	No. of farms		
Qalyubia	17	3	11
Dakahlia	3	1	2
Gharbia	2	0	0
Total	22	4	13

4. DISCUSSION

Mycoplasma infections are of high economic importance in the poultry industry because high mortality rates, poor carcass conditions and loss of egg production. Avian *mycoplasmas* are induced after the host is affected by other disease-causing agents such as bacteria and viruses and/or after an episode of host weakness (Yoder et al.,1991) Interestingly, the classical microbiological techniques currently in use for *Mycoplasma* detection and identification are not satisfactory in most situations but remain necessary for drug susceptibility testing. The complexity associated with them makes alternative approaches more attractive (Anbazhagan et al., 2010).

In our study, from 22 examined poultry farms and by conventional identifications methods, *Mycoplasma* was recorded in 4 farms (18.18 %).Yoder (1984) stated that *Mycoplasma* were fastidious. They were more sensitive than bacteria to environmental conditions such as tonicity of the medium and the exposed plasma membrane was sensitive to damage by surface active substance. This explains the decreased chances for isolation of *Mycoplasma* by conventional methods.

M. gallisepticum was isolated from chickens showing signs of chronic respiratory disease with a percentage of 5% (Abd El Aziz *et al.,* 2007). Meanwhile, Hassan (2001) isolated *M. gallisepticum* with an incidence rate 14.6%. Also, Heleili *et al.* (2011) isolated *M. gallisepticum* from respiratory organs of chickens with a percentage of 21.67%.These results are agreed with our detection result (18.18%)which confirm low detection rates for *Mycoplasma* by conventional methods due to loss of extra *mycoplasma* cells during cultivation and cell membrane damages in comparison to PCR results.

PCR was used to assess the prevalence of microorganisms incriminated in occurrence of CRD signs in poultry, and the results were compared with those obtained using culture techniques. Obviously, the PCR assays have demonstrated a significantly higher rate of detection of *Mycoplasma* in poultry farms with various problems in Egypt than detection by classical culture procedures. Overall, PCR could detect *Mycoplasma* in 13 farms (59.09%). Also, Marois *et al.* (2002) recorded positive *M. gallisepticum* cases through culture identification 3.75% in comparison to molecular technique (42.4%).

Moreover, Rauf *et al.* (2013) used *16S* rRNA gene as species specific primers of *MG* and found overall 27.6% from field birds were positive for *MG* by conventional cultivation methods in comparison to PCR (68.94%). These results are attributed to the fact that PCR can detect DNA from both viable and non-viable bacteria and hence is more reliable diagnostic test in terms of sensitivity and specificity.Thus, the use of reference genes differ from genes applied in the previous studies, the results are similar to our results and *Mycoplasma* showed different isolation results for MG and MS at the same sample.

In a previous study, Boussetta *et al.,* (1997) isolated *MG* from 15 flocks (23.8 %), while *M.S* was isolated from only five flocks (7.9 %) in Tunisia. The prevalence of *M.S* in backyard chickens averaged between 68.6 % and 100%, while the prevalence of *M.G* was averaged between 32.8% and 55.1% (Xavier et al., 2011).But in our study, overall, 5 out of 22 examined farms were assigned to be positive for *M. synoviae*(22.72%) were with single infection, 3 (13.63%) associated with *M. gallisepticum* and 5 (22.72) showed mixed infections, meanwhile 9 farms only (40.9%) were negative.

Multiplex PCR assay was optimized for successful detection of genes with expected amplicon sizesfrom clinical specimens collected from suspected farms. The results showed that mPCR yielded a detectable DNA fragment of expected molecular weight only in the presence of their respective DNA template and gave negative results when tested with other bacteria.

Siddique *et al.* (2012) optimized the multiplex PCR for successful detection of five of the respiratory tract pathogens including *M. gallisepticum*, *M. synoviae*, Newcastle disease virus, Infectious bronchitis virus and Avian influenza virus. Bayatzadeh*et al*. (2011) amplified the conserved region of 16S rRNA gene for the detection of *Mycoplasma* genus in 163bp fragment and *M. synoviae* in 207bp.

In the current work, two reference strains of *Mycoplasma* including *M.synoviae* (*vlhA*)and *M. gallisepticum (mgc2)* with expected amplicon sizes 396bp and 300bp respectively , which amplified using its respective primer pairs.

In general, the use of multiplex PCR reactions for groups of organisms causing similar syndromes provides an efficient way to ask several related epidemiological questions simultaneously. On the other hand, vaccination didn`t give complete protection against infection, but some were effective in suppress the multiplication of the organism, resulting in less tissue damage followed by faster recovery (Hildebrand *et al.*,1983; Rodriguez and Kleven, 1985).

Antibiotic treatment (chemotherapy) is necessary in complement of biosecurity to control *Mycoplasma* infections. It is logic that for a successful and aimed *mycoplasma* infection treatment, it is necessary to have regular antibiogram tests of *M. gallisepticum* and or *M. synoviae* in the field for monitoring susceptibility of *Mycoplasma* prevalent in the farms. In previous studies, Mycoplasma was reported to show sensitivity *in vitro* and *in vivo* to tetracyclines and quinolones (Jordan and Horrocks 1996;Bébéar et al., 1999; Wu et al., 2000)which is opposite to our results. On the other hand, our results came in agreement with that reported by Whithear et al. (1983), who recorded that *Mycoplasma* isolates showed resistance to oxytetracycline and erythromycin.

5. CONCULSION

M. synoviae consider as the most common poultry *mycoplasma* causes respiratory infections in poultry. Higher prevalence of mixed M. *synoviae* and M. *gallisepticum* infections in poultry with respiratory manifestations was recorded. Multiplex PCR is sensitive and specific for simultaneous detection of *M. synoviae* and *M. gallisepticum* in clinical specimens in a single reaction. *Mycoplasma* are highly sensitive to gentamicin and nitrofurantoin antibiotics, while they are highly resistant to tetracyclines

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